

INDUCIBLE PROMOTERS

Ins A1
Ins A2
The present invention relates to novel inducible promoters derived from plants and their application in the controlled expression of heterologous genes.

5 *Ins A3*
The aim of crop plant genetic engineering is to insert a gene (or genes) which alter a plant's characteristics without altering otherwise desirable elements of the genotype of the original plant. Thus, the genetic make-up of crop plants can be extended to include genes outside of the original genetic pool which are not accessible by traditional crop
10 breeding techniques. One important aspect of such modification is the choice of promoter. Conceptually, identification and characterisation of promoters allows the possibility to construct chimeric genes in which a promoter from one gene can be used to drive the expression of a protein encoded by a separate gene under conditions and at sites within the plant specified by the promoter.

15 Promoters often contain elements which are recognised by inducible factors which regulate the temporal and tissue-specific expression of genes. These elements are typically short sequences and are found in promoters of many (if not all) genes which respond to the same signal. Thus, in plants, analysis of promoters of genes which are
20 upregulated by the phytohormone abscisic acid have identified a common element CCACGTT within these promoters (Marcotte *et al.*, *Plant Cell* 1 969-976 (1989) and Pla *et al.*, *Plant Mol. Biol.* 21 259-266 (1993)). Similarly, promoters responsive to ethylene contain the PR box AGCCGCC (Broglie *et al.*, *Plant Cell* 1 599-607 (1989) and Deickman *et al.*, *Plant Physiol.* 100 2013-2017 (1992)). Furthermore, possession
25 of a selection of such response elements may confer upon a promoter either the ability to drive gene expression in response to any one of several signals or to display synergistic enhancement of expression in response to several signals. Table 1 lists a number of consensus response element sequences identified in plants.

Table 1

Promoter Response Elements

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Sub B₂

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Name	Sequence	Sensitivity
ABRE	CCACGTT	ABA
DRE1	TACCGACAT	Drought
E-8	ATAAGGGGTTGGT	
G Box	GTGTCAC	
H Box	GGTAGG	
JA Box	CCCTATAGGG	JA?
Myb	TGGTTA	
Myc	CANNTG	
PR Box	AGCCGCC	Ethylene
TCA	TTATCTCCTT	

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Thus, from the DNA sequence of promoters, it is possible to predict the circumstances under which a promoter will be expressed by looking for already identified response elements within its sequence. It is with such inducible promoters that the present application is concerned.

Previously the expression of foreign proteins in transgenic plants has been driven by a 'constitutive' promoter such as Cauliflower mosaic virus 35S (CaMV 35S). Use of such a promoter in a commercial application is limited where the promoter is being used to drive toxic protein synthesis, or proteins which impose a substantial metabolic burden on the plant. These problems can be overcome if expression of the target protein is under the control of an inducible promoter, either just prior to or just after harvesting.

Several plant derived promoters have been previously proposed as being suitable for chemically regulated transgene expression (Gatz, C (1997) *Annu. Rev. Plant Physiol. Plant. Mol. Biol.* **48**, 89-108). Such promoters are inducible by safeners or elicitors or chemicals mediating wound responses or chemicals inducing systemic acquired resistance (SAR). Promoters induced by safeners or by chemicals that induce SAR have been most studied. For example WO 93/01294 and Jepson *et al.*, (1994) *Plant Mol. Biol.* **26**, 1855-1866, describe the isolation of a glutathione S-transferase gene (GST-27) inducible by the safener N,N-diallyl-2,2-dichloroacetimide. The applications of this promoter are however limited since the promoter is constitutively expressed in roots.

The phenomenon of systemic acquired resistance (SAR) following infection of a plant with pathogenic micro-organisms has long been established. When a plant is invaded by a potential pathogen, which it is able to recognise, a resistance response is activated. This response, known as an incompatible interaction, typically involves hypersensitive cell death at the site of pathogen ingress, phytoalexin synthesis, the production of active oxygen species, cell wall strengthening, local induction of defence genes and salicylic acid (SA) accumulation. Following on from this local response, SAR is established throughout the plant. SAR endows uninfected tissue with the ability to respond more rapidly to further infection and this resistance is effective against a wide range of potential pathogens. The synthesis of a number of relatively unrelated proteins known as pathogenesis-related proteins (PR proteins) accompanies the onset and establishment of SAR.

It has long been known that treatment of plants with either SA or acetyl-SA (aspirin) can induce resistance to pathogens (White, R.F. (1979). *Virology* **99**, 410-412). Recent research has demonstrated that SA plays a key role in both the local and systemic induction of PR proteins and the establishment of SAR (Malamy *et al.*, (1990) *Science* **250**, 1002-1004; Metraux *et al.*, (1990) *Science* **250**, 1004-1006; Yalpani *et al.*, (1991): *Plant Cell* **3**, 809-818). Furthermore, SAR and PR protein accumulation are

compromised in plants that constitutively express a bacterial salicylate hydroxylase (which converts SA to catechol) further supporting a role for endogenous SA in these processes (Gaffney *et al.*, (1993) *Science* **261**, 754-756). Spraying, injection or root-feeding plants with SA strongly induces expression of PR gene promoters by 50-1000-
5 fold over basal levels (e.g. Mur *et al.*, (1996) *Plant J.* **9**, 559-571). However, since the concentration of SA used in these studies (typically 1-2mM) is phytotoxic, a great deal of effort has been put into identifying less harmful compounds capable of mimicking SA.

10 One compound in particular, BTH (benzo(1,2,3)thiadiazole-7-carbothoic acid S-methyl ester), is already marketed as a 'crop enhancer' and is available for large scale use in the field (Gorlach *et al.*, (1996) *Plant Cell* **8**, 629-643). An aqueous solution of 1.2 μ M BTH is sufficient to induce PR gene expression (Friedrich *et al.*, (1996) *Plant J.* **10**, 61-70). Commercial preparations of BTH are sufficient to induce very strong PR
15 gene expression in all plants tested including arabidopsis and wheat.

Gene induction following spraying with BTH is maximal 2 days after application and persists for at least 10 days. Although it induces enhanced resistance in treated tissue, its mode of action is unknown, nor is it known whether this compound can mimic all
20 of SA's effects such as potentiation of gene induction or the pathogen-induced oxidative burst.

Thus, a potential source for inducible promoters is the pathogenesis-related (PR) 'family' of defence-related genes. PR genes are a diverse set of proteins some of which
25 (e.g. PR-2 and PR-3 classes) have known functions as chitinases or beta-1,3-glucanase. Others (e.g. the PR-1 and PR-5 classes) are induced during plant-pathogen responses but have no clearly identifiable function. Typically, PR proteins of each class contain members with acidic or with basic pHs. Although there are exceptions to the rule, basic PR proteins tend to be localised to an intracellular site (e.g. the vacuole)
30 whilst acidic PR proteins are secreted.

Plants also have to respond to a variety of other environmental stresses including water stress, mechanical and herbivore wounding, UV light and oxidative stress, and both high and low temperatures. PR genes are upregulated in a number of these conditions. Thus, expression of tobacco osmotin (a basic, vacuolar PR-5 gene) is induced not only by pathogen challenge but also by salt stress (Grillo *et al.*, *Physiologia Plantarum* **93** 498-504 (1995)). PR-1a expression is induced following treatment with hydrogen peroxide (which induces oxidative stress) and in plants subjected to UV stress (Yalpani *et al.*, *Planta* **193** 372-376 (1994)). The responses to wounding and to pathogen challenge share a number of similar features including expression of defence genes and the establishment of a systemic response mediated by mobile signals. As a rule, basic PR proteins are also responsive to wounding stimuli.

A number of elements present in PR gene promoters have been identified. The PR-2d gene (encoding a β -1,3-glucanase) from tobacco is expressed in tissue undergoing hypersensitive response (HR) following tobacco mosaic virus (TMV) challenge and is induced by exogenous SA (Shah *et al.*, *Plant J.* **10** 1089-1101 (1996)). Region -364 to -288 in the PR-2d promoter confers SA sensitivity and a 25 bp element in this region is recognised by nuclear factors from tobacco. An SA responsive element has also been isolated from the CaMV 35S promoter at position -90 to -46. The element corresponds to an as-1 site (Qin *et al.*, *Plant Cell* **6** 863-874 (1994)). The sequence TCATCTTCTT is repeated several times in the barley β -1,3-glucanase promoter and is present in over 30 stress-induced genes (Goldsbrough *et al.*, *Plant J.*, **3**(4):563-571 (1993b)). This region binds 40 kDa tobacco nuclear proteins, the binding of which is increased in SA-treated plants. Buttner *et al.*, *Proc. Natl. Acad. Sci. USA* **94** 5961-5966 (1997) have shown that Arabidopsis ethylene responsive element binding proteins bind to the PR box and that PR- and G-boxes exhibit synergistic effects.

PR-1 genes have been studied in some detail and the promoter of one, tobacco PR-1a, has been proposed as a suitable inducible promoter (EP 0 332 104 A2). Tobacco PR-

1a is expressed both locally in infected tissue and later during establishment of SAR. Thus, infection of Samsun NN tobacco plants leads to accumulation of endogenous PR-1 proteins in both inoculated leaves (approx. 4 days after infection) and later (approx. 8days) in upper uninfected leaves on the same plant. Local (approx. 12 hours post-inoculation) and systemic (3-7 days) induction of PR-1a-GUS expression in *Pseudomonas syringae* pathovar *syringae*-infected tobacco has been reported. (Bi *et al.*, (1995) *Plant J.* 8:235-245; Mur *et al.*, (1996) *Plant J.* 9:559-571). Direct application of SA induces high levels of PR-1a promoter-GUS expression in transgenic tobacco [Bi *et al.*, supra]. The SAR inducers BTH and INA also induce high levels of both endogenous PR-1a and PR-1a-GUS expression.

Wounding also induces a slight increase in PR-1a-GUS expression (Darby, R., unpublished observations, Ohshima *et al.*, (1990) *Plant Cell* 2, 95-106). As with other PR-1 proteins, PR-1a exhibits developmental expression. Thus, PR-1a-GUS is expressed in leaves, petioles, stem cortex, pollen and sepals of flowering tobacco (Uknes *et al.*, *Plant Cell*, 5(2):159-169 (1993)). PR-1a-GUS is also expressed in roots (Kenton, P; unpublished observations).

The PR-1a promoter has been studied extensively. Van de Rhee and Bol (*Plant Mol. Biol.*, 21(3):451-461 (1993b)) identified four regulatory elements in the PR-1a promoter all of which were required for maximal activity and no single element of which was capable of conferring promoter activity. The PR-1a promoter contains a number of elements that bind GT-1-like and Myb1 transcription factors (Buchel *et al.*, *Plant Mol. Biol.*, 30(3):493-504 (1996)). In addition, SA and active analogues induce expression not only of PR genes but also myb1.

The high level of sensitivity to SA shown by the PR-1a gene and the very high levels of PR-1 α -GUS expression following SA treatment or infection could lead to inappropriate expression of any PR-1a promoter-gene fusion as a result of perturbation

of endogenous SA levels (brought about, e.g., by a change in redox status). This may limit its usefulness in driving genes the products of which are either toxic at high levels or impose a substantial metabolic burden on the plant. Finally, PR-1 genes in general and PR-1a in particular show a high level of constitutive/developmental expression, especially during flowering. Again, this could lead to a high degree of unscheduled expression of PR-1a promoter-driven transgenes.

PR-5 proteins are another class of PR proteins, and can be divided into two groups, the acidic extracellular thaumatin-like proteins and the basic intracellular osmotins.

Classically osmotins have been associated with abiotic stresses. However, this osmotically-induced expression is typically additional to a high degree of constitutive (Stintzi *et al.*, *Biochimie*, **75**:687-706 (1993); Leone *et al.*, *Plant Physiol.*, **106**:703-712 (1994); Van Kan *et al.*, *Plant Mol. Biol.*, **27**:1205-1213 (1995)) and developmental expression (Linthorst, *Crit. Rev. Plant Sci.*, **10**:123-150 (1991); Stintzi *et al.*, *Physiol. Mol. Plant Pathol.* **38** 137-146 (1991); Raghothama *et al.*, *Plant Mol. Biol.* **34**:393-402 (1997)). Osmotin expression is also elevated in response to stresses such as desiccation, wounding, low temperature (Raghothama *et al.*, *Plant Mol. Biol.* **23**:1117-1128 (1993); Grillo *et al.*, (1995) *supra*; Zhu *et al.*, *Plant Mol. Biol.*, **28**:17-26 (1995b)), and chemical factors such as ethylene (in tobacco Raghothama *et al.*, 1993, *supra*; Chang *et al.*, *Physiologia-Plantarum*, **100**:341-352 (1997)), and cytokinins (Thomas & Bohnert, *Plant Physiol.*, **103**:1299-1304 (1993)). Pathogen challenge also induces osmotin expression (Zhu *et al.*, *Plant Physiol.*, **108**:929-937 (1995a); (1995b), *supra*; Chang *et al.*, (1997), *supra*) which may be systemic for some osmotins (Zhu *et al.*, (1995b), *supra*) or local for others (Zhu *et al.*, (1995a), *supra*). Thus the osmotin genes do not appear to be ideal sources of inducible promoters.

Unlike the vacuolar-localised osmotins, acidic PR-5s (aPR-5) are secreted and lack the C-terminal extension which may be a vacuolar targeting signal (Linthorst, (1991), *supra*; Stintzi *et al.*, (1993), *supra*). aPR5 proteins have been shown to be accumulated on pathogen attack, for example in barley (Bryngelsson & Green, *Plant Mol. Plant*

Path., 35:45-52 (1989); Boyd *et al.*, *Plant Mol. Plant Path.*, 45:47-58 (1994); Reiss & Bryngelsson, *Physiol. Mol. Plant Path.*, 43:331-341 (1996); Schweizer *et al.*, *Plant Physiol.*, 114:73-88 (1997); Vale *et al.*, *Physiol. Mol. Plant Path.*, 44:207-215 (1994)) and wheat (Rebmann *et al.*, *Plant Mol. Biol.*, 17:283-285 (1991)). Using western blots, 5 Stintzi *et al.*, (1991) were unable to detect aPR-5 in healthy tobacco leaves whereas osmotin was constitutively expressed. Following challenge with TMV, aPR-5 appears after 4-6 hours, whereas osmotin begins to accumulate over basal levels 2-4 hours post-inoculation (Stintzi *et al.*, 1991). aPR-5 has been localised to extracellular pocket-like structures between mesophyll cells close to the infection site in TMV-infected 10 tobacco (Dore *et al.*, *Arch. Virol.*, 120:97-107 (1991)).

A number of other treatments have been shown to induce expression of extracellular aPR-5 proteins. Sunflower extracellular aPR-5 proteins are induced in leaf discs by 5mM aspirin, 10mM ethephon, 10mM NAA, 10mM 2,4 D, UV light, 5mM MnCl₂, 15 5mM HgCl₃, 5mM citric acid and 5mM oxalic acid (Jung *et al.*, *Journal of Plant Physiol.*, 145:153-160 (1995)). 1ppm INA induces expression of a barley homologue of rice thaumatin-like protein and JA that of a barley aPR5 (Schweizer *et al.*, (1997), *supra*). aPR-5s are also expressed in cold-acclimated winter rye where they may play a role in preventing ice damage (Hon *et al.*, *Plant Physiol.*, 109:879-889 (1995)).

20 However, information concerning developmental expression of aPR-5s is limited. In maize constitutive expression is mainly confined to non-embryonic tissues of the developing seed peaking two to four weeks after pollination but still detectable in desiccated seed. Only slight expression was detectable in maize leaves (Malehorn 25 *et al.*, *Plant Physiol.*, 106:1471-1481 (1994)). A 29 kDa thaumatin-like protein has been detected in ripe cherry fruits (Fils-Lycaon *et al.*, *Plant Physiol.*, 111:269-273 (1996)).

30 Little is known about aPR-5 promoters since only a tobacco aPR5 promoter from the E2 gene has been isolated, fused to the reporter gene GUS and analysed (Albrecht

et al., (1992) Plant Mol Biol. 18, 155-158). This study showed that TMV induced both local and systemic GUS activity; the local response being greater than the systemic response. The element(s) responsible for this TMV induction of this aPR-5 were found to lie in the -1364 to -718 promoter region. By nucleic acid hybridisation no significant homology was found between this tobacco PR-5 promoter and the PR-1a promoter.

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Ins a 4

The present invention is based on the discovery of novel and useful inducible promoters which overcome some or all of the problems associated with the prior art by providing an inducible promoter which is responsive to low levels of an environmentally-acceptable and non-phytoxic inducing agent capable of use in both field and *in vitro* conditions, and which also exhibits a low level of environmentally- or developmentally-induced expression, and low levels of pathogen induced systemic activation, when compared with similar promoters.

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Ins a 5

Thus, in a first aspect of the present invention there is provided a recombinant or isolated DNA molecule comprising an inducible gene promoter which:

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- i) naturally drives the expression of a 21.3kDa protein in *Asparagus officinalis* upon induction by plant regulators; or
- ii) naturally drives the expression of proteins equivalent to the 21.3kDa protein of *Asparagus officinalis*, from the *Lillaceae* or *Amaryllidaceae* families; or
- iii) naturally drives the expression of proteins substantially homologous to those of i) or ii); or
- iv) hybridises under stringent conditions to any one of the promoters of i), ii) or iii).

The promoter defined in i) is derived from an *Asparagus officinalis* thaumatin-like PR-5-related gene (AoPRT-L) and is able to drive expression of heterologous genes in dicots and monocots. pAoPRT-L has several advantages over the use of previously described promoters in the expression of heterologous genes. Table 2 compares the characteristics of the AoPRT-L promoter with that of PR1a and osmotin.

Table 2 - Comparative Expression Patterns of PR-1a, Osmotin and AoPRT-L

	<u>Treatment</u>	<u>Site</u>	<u>PR-1a</u>	<u>Osmotin</u>	<u>AoPRT-L</u>
5	None	Leaves	+ (flowering)	+	-
		Stem	+ (vasculature +) (leaf axil)	+	+ (leaf axil)
		Petiole	+ (flowering)	?	+ (leaf axil)
		Roots	+	+	+/-
		Flowers	+ (sepal+pollen)	?	+ (sepal tip)
10	Pathogen	HR lesion	+	?	+ (TMV)
		Local	+	+	+
		Systemic	+	+/-	-
15	BTH		+	?	+
	INA		+	+	+
	Wound		+/-	+	-

20 From this data, and data in the Examples, it can be seen that:-

- 1) pAoPRT-L exhibits minimal developmentally-regulated expression.
- 2) pAoPRT-L, unlike tobacco pPR1a and aPR5-E2, is not systemically activated by pathogen infection.
- 25 3) pAoPRT-L is not responsive to ABA, ethylene, oxidative and osmotic stresses and wounding.
- 4) pAoPRT-L expression is induced by SA and by BTH (Novartis) a chemical that is licensed for field use.

30 These characteristics make the pAoPRT-L promoter a favourable candidate for use in expression of foreign proteins in transgenic plants. In addition, suitable promoters as defined in ii) or iii) may also be identified which drive the expression of pathogenesis related proteins equivalent or homologous to the pAoPRT-L protein of

Asparagus officinalis in the *Lillaceae* or *Amaryllidaceae* family or indeed in other plant families.

Proteins substantially homologous to the AoPRT-L protein of *Asparagus officinalis* or equivalent proteins from the *Lillaceae* or *Amaryllidaceae* families may be readily identified by a person skilled in the art using techniques known in the art, for example as described herein. Such proteins are those which are functionally equivalent to the AoPRT-L protein. Thus, substantially homologous proteins are preferably inducible pathogenesis related proteins which are substantially free of systemically activated expression and developmentally regulated expression.

An important advantage of the inducible promoters of the present invention is the lack of developmentally regulated and systemically activated expression. This is in contrast to the constitutive promoters usually used to drive expression of heterologous genes in transgenic plants, or the inducible promoters which also become activated in a developmental manner, or throughout the plant as a result of pathogen invasion. The use of an inducible promoter which is not developmentally or systemically activated is particularly useful in the production of transgenic gene products from plants in field conditions, as it allows the controlled harvesting of the desired product. A promoter which is not developmentally regulated will allow the expression of genes whose products may be harmful to, or reduce the fitness of the plant. If such products were expressed constitutively either in the entire plant or in a significant portion of the plant, or at key stages of development, the plant may suffer, develop abnormally or die. For example, promoters such as GST27 and PR1a have significant developmental expression which limits the range of transgenes that can be expressed using these promoters. However, where an inducible promoter of the present invention is used, any gene may be expressed because the risk of gene expression at inappropriate stages of development is avoided. The use of a promoter of the present invention which is further not systemically activated in response to

pathogen invasion also broadens the spectrum of genes which may be expressed from the promoter because inappropriate expression, which may be harmful to the plant, is avoided in field conditions where plants are highly susceptible to pathogen attack. In summary, and as a result also of the lack of activation in response to stimuli such as oxidative and osmotic stresses, ABA, ethylene and wounding, the AoPRT-L promoter offers improved controllability of expression over currently existing plant derived promoters which are used or will potentially be used for chemically induced transgene expression. The inducibility of the promoter with a chemical accepted for field use further emphasises the suitability of the AoPRT-L promoter for expression of heterologous genes in field plants.

Pathogenesis related proteins, or PR proteins, may for the purpose of the present invention, be defined as those proteins which are expressed in plants reacting to pathogens. Hypersensitivity to a pathogen is characterized by a local response which includes necrosis of tissues immediately surrounding the infected site. Other features of a local hypersensitive response include phytoalexin synthesis, production of active oxygen species, cell wall strengthening, local induction of defence genes and accumulation of salicylic acid. This is in contrast to a sensitive response in which the pathogen spreads throughout the whole plant. The hypersensitive local response may be followed by the induction of systemic acquired resistance (SAR). This allows uninfected tissues to respond quickly upon re-invasion of a pathogen. Pathogenesis related proteins may be expressed during the hypersensitive response, and upon the onset of SAR. Examples of pathogens which may result in the expression of one or more of the pathogenesis related proteins include viruses or viroids, for example tobacco or cucumber mosaic virus, ringspot virus, necrosis virus, pelargonium leaf curl virus, red clover mottle virus and other similar viruses, fungi, for example, *Phytophthora parasitica* or *Peronospora tabacina*, bacteria such as *Pseudomonas syringae*, or *Pseudomonas tabaci*, or aphids such as *Myzus persicae*. It should be understood that this is not an exhaustive list, and the

hypersensitive response and SAR may be induced by a number of other pathogens not listed here.

A pathogenesis related protein may be identified by any number of known techniques. For example, a PR protein may be identified by comparison of proteins isolated prior to, and at stages during and after, pathogen infection. PR proteins may also be identified by homology to known PR proteins, promoter analysis, or functional analysis of the expression products of a cDNA library. These techniques, together with other suitable techniques, would be known to a person of skill in the art.

For the purpose of the present invention, systemic activation of a gene may be defined as the activation of a gene prior to or during the systemic acquired resistance response of a plant to pathogen resistance. As discussed above, such genes will typically be expressed in uninfected areas of a plant, more typically throughout the plant, following the local response of a plant as a result of pathogen infection. Such genes usually encode products involved in the systemic acquired resistance of a plant to pathogen invasion, for example pathogenesis related proteins. Conversely, a gene which is substantially free of systemic activation will be under the control of a promoter which does not activate expression of the gene throughout the plant in uninfected tissues that are distant to the site of pathogen attack. Expression of such genes is limited to the site of infection or immediately adjacent to the site of infection. Promoters which are substantially free of systemic activated expression do not give rise to levels of expression throughout the plant prior to, during or after the systemic acquired resistance response, which are significantly above basal levels of expression, and do not give rise to expression throughout the plant that is a significant fraction of the expression levels attained locally in areas at or adjacent to

sites of infection. Such basal levels may vary from plant to plant, although ideally should approach or be zero.

5 The cells of any particular plant will all contain the same genome, and thus behave according to the same rules. However, cells of a plant may exist in a variety of different states depending on the developmental pathway which they have followed. A number of genes are involved in the control of developmental choices made by plant cells. These genes, known as developmental control genes, are
10 developmentally regulated, and typically expressed only in a particular plant tissue or organ at a specific stage of development. For example, a gene involved in the control of development of the flowering organs of a plant will only be expressed in those tissues from which flowering organs develop, prior to or during flowering. A gene which is substantially free of developmentally regulated expression may be defined as one which is not expressed only in a particular organ or tissue at a
15 specific stage of development, or is expressed in such tissues at such times at a basal level only. Genes which are substantially free of developmentally regulated expression may be expressed in most tissues, throughout the life of the plant, for example those genes involved in food production, transport and storage. Other genes which are substantially free of developmentally regulated expression may be
20 expressed only in response to external stimuli, such as environmental and chemical stimuli. Developmentally regulated genes and their promoters may be identified in a number of ways. For example, mutation analysis will allow the identification of mutations which prevent or inhibit the development of a particular organ or tissue of a plant, and thus the corresponding promoters and genes which must be involved in
25 regulation of development. Such promoters and genes may also be identified from cDNA, genomic DNA or mRNA libraries, or by nucleotide or amino acid sequence homology to known developmentally regulated genes, or homology between the upstream regulatory sequences.

A promoter which is substantially free of developmentally regulated expression, in accordance with the present invention, may be identified in a number of ways. For example, mutation or deletion of a promoter which is substantially free of
5 developmentally regulated expression will not result in inhibition or prevention of development, or disruption of the plant body plan.

Preferably, the recombinant or isolated DNA of the present invention encodes a promoter which is further substantially free of activation in response to
10 environmental and hormonal stimuli, such as ABA, ethylene, oxidative and osmotic stresses and wounding. As a result, inappropriate expression in response to the above stimuli of a gene under control of the promoter of the invention is avoided. In this way, controlled expression of a gene is possible. Promoters which are substantially free of activation in response to environmental or hormonal stimuli may
15 be identified by a number of ways, for example by linkage of the promoter sequence of interest to a reporter gene, transfection of a plant with the promoter-reporter gene construct, and analysis of the expression of the reporter gene following application of a hormonal or environmental stimuli. Promoters which do not activate expression of the reporter gene in response to the above stimuli, or cause low or basal levels of
20 the reporter gene may be identified as being substantially free of chemical or environmental stimulation. Acceptable levels of low or basal levels will depend entirely on the gene being expressed. Thus, if the gene encodes an innocuous product, a low level of constitutive expression will be unlikely to impose a great metabolic burden on the plant. However, if the product is phytotoxic, even a low
25 level of expression may be deleterious.

Preferably, the recombinant or isolated DNA of the invention encodes a promoter which is inducible in response to plant regulators. The plant regulators are

preferably inducers of SAR, and may be natural or synthetic. Examples of such SAR inducers include salicylic acid (SA) and BTH (Novartis), and the analogues such as 4-chloro-SA, 5-chloro-SA and 3,5-chloro-SA, benzoic acid (BA), 2,3-dihydro-BA, dichloroisonicotinic acid (INA) and a number of halides of this compound (Sanchez-Casas & Klessig, *Plant Physiol.* **106** 1675-1679 (1994); Conrath *et al.*, *PNAS* **92** 7143-7147 (1995)).

In the present invention, the gene encoding the 21.3 kDa pathogenesis related protein in *Asparagus officinalis* and equivalent proteins in the *Lillaceae* or *Amaryllidaceae* families will be referred to as the AoPRT-L gene. AoPRT-L shares a degree of homology with other PR proteins, particularly with those of the PR-5 family, such as tobacco osmotin and osmotin like protein. Several of the PR-5 family proteins have been characterized and some, for example osmotin, have been shown to exhibit local and systemic activation upon pathogen invasion and developmentally regulated expression. In contrast, the acidic PR-5 gene products appear to show local activation in response to pathogens. Very little information is available regarding the developmental expression of these genes, although evidence suggests that they, too, are developmentally regulated. For example, constitutive expression has been observed in non-embryonic tissue of the developing seed in maize (Malehorn *et al.*, 1994 *Plant Physiol.*, **106** : 1471-1481, and a thaumatin-like protein has been observed accumulating in ripening cherry fruits (Fils-Lycaon *et al.*, 1996). The transcription profile of the AoPRT-L gene differs from these genes in that it is not activated in the systemic response to pathogen invasion, and exhibits minimal developmentally regulated expression. Further, unlike the PR-5 genes, AoPRT-L is not induced in response to a wide range of stimuli including ABA and ethylene. Salicylic acid and its synthetic analogue BTH both have the ability to induce expression from the AoPRT-L promoter.

The molecular weight of the AoPRT-L protein given herein is merely putative, and derived from the number of amino acids present in the protein. The 21.3 kDa protein encoded by the AoPRT-L gene has 223 amino acids. The molecular weights refer to the unmodified protein, and do not take account of any changes as a result of post-translational modifications.

The molecular weight given above refers only to that of the AoPRT-L gene of *Asparagus officinalis*. Those skilled in the art will be readily able to identify equivalent proteins from the *Liliaceae* or *Amaryllidaceae* families using standard methods known in the art. For example, genes encoding the proteins equivalent to AoPRT-L may be identified by nucleic acid hybridisation studies, Restriction Fragment Length Polymorphism mapping, PCR cloning, and other known methods. The AoPRT-L gene or fragments thereof may be used as a probe to identify genes or DNA sequences encoding equivalent proteins. A fragment of the AoPRT-L gene may be 10, 20, 30, 50, 75 or 100 nucleotides. Preferably, a fragment of 15 to 20 nucleotides is used as a probe. Typically, the probe will be used to hybridize to genes encoding equivalent proteins, under stringent conditions. Suitable conditions may be those given in Plant Genetic Transformation and Gene Expression: A Laboratory Manual, Ed. Draper, J *et al*, 1988, Blackwell Scientific Publications pp252-255 (which is hereby incorporated by reference), modified as follows: prehybridization, hybridization and washes at 55-65°C, final washes with 0.5X SSC, 0.1% SDS omitted.

Preferred promoters of the present invention are those which drive expression of the 21.3kDa protein or equivalent proteins of the *liliaceae* or *Amaryllidaceae* families. The sequence of the *A. officinalis* promoter is shown in Figure 6. It is envisaged that the whole promoter sequence shown in Figure 6 may be used, or fragments thereof. The fragments may be 20, 50, 100 or 150 nucleotides in length. Such

fragments are those which retain the characteristics of the native promoter sequence, namely being substantially free of systemically activated or developmentally regulated expression. Preferably, such fragments will also be inducible by SA or BTH. The isolation of the promoter sequences shown in the figures will be described in the Examples.

The promoter of the AoPRT-L gene from *Asparagus officinalis* may be isolated from a plant using techniques known in the art. For example, the promoter may be isolated by i) synthesizing cDNA from the mRNA isolated from cultured, mechanically isolated *Asparagus officinalis* cells, ii) differentially screening the cDNA to identify those clones induced upon adaptation to cell culture conditions, iii) isolating a differentially expressed cDNA encoding a gene of interest, iv) using this cDNA to probe genomic DNA of *Asparagus officinalis* for the sequence encoding the gene of interest v) identifying the upstream regulatory regions of the gene of interest, which includes the promoter of the gene. The promoters of equivalent proteins from the *Lillaceae* and *Amaryllidaceae* families, and promoters of proteins substantially homologous to the proteins of the first aspect of the invention may be identified using standard techniques known in the art. For example, the promoter may be isolated by i) synthesizing cDNA from mRNA of a plant cell of interest; ii) screening the cDNA library to identify clones showing the appropriate expression pattern; iii) isolating the cDNA clone of interest; iv) using this cDNA to isolate the gene by screening a genomic library;; v) identifying the upstream regulatory elements of the gene.

The techniques used in the above steps are known in the art. The isolation of viable cells from a plant according to step i) is described in International Patent Application WO 93/05164. Briefly, viable cells may be sheared from any monocotyledon or dicotyledon. Cells isolated in this manner from *Asparagus officinalis*, and placed in

growth medium will dedifferentiate and initiate cell division when placed in cell culture. The adaptation to culture conditions results in the expression of a large number of genes which may not usually be expressed. Because the induction of cell division and dedifferentiation are features usually associated with wound response phenomena in dicots, it is envisaged that the promoters of the present invention
5 isolated from the monocot *Asparagus officinalis* may be used to induce gene expression in either dicots or monocots.

The change in gene expression upon culturing of the mechanically isolated cells
10 makes them a rich source of gene transcripts, and thus suitable for the production of a cDNA library. The construction and differential screening of a cDNA library is described in WO 93/05164.

Optionally, the suitability of promoters which hybridise to the above promoters of
15 the present invention may be further assessed by functional analysis. Thus, favoured promoters which hybridise to the above promoters are those which do not show substantial systemic or developmentally regulated activation. Promoter sequences which hybridise under stringent conditions to whole or part of the promoter sequences of pAoPRT-L of *Asparagus officinalis*, equivalent proteins from the
20 *Lillaceae* or *Amarydillaceae* families, or proteins substantially homologous thereto, are also included within the scope of this invention.

For identification of substantially homologous proteins or promoters, one can make use of specialist computer programmes. For instance one can make use of the default
25 parameters of the GAP programme of the GCG package available on the SEQNET Computational Molecular Biology Facility at SERC, Daresbury, UK. This program provides "scores" for % identity and % similarity. Preferably, sequences having 60% or greater identity or 65% or greater similarity are included within the scope of

the present invention. Thus, sequences having 70%, 80%, 90%, 95% or indeed 99% identity are included within the scope of the present invention. The skilled person will appreciate that these limits apply to both nucleic acid sequences and amino acid sequences (when one is identifying a protein analogous to the protein identified in the present invention, for instance). When considering sequences at the nucleic acid level, it is generally the case that identity/similarity of the coding sequence and/or promoter will be assessed.

Such promoter sequences may be identified by using standard techniques known in the art. For example, the pAoPRT-L promoter, or promoters of equivalent proteins from the *Lillaceae* or *Amaryllidaceae* families, or fragments thereof may be used as probes to identify promoters which will hybridise thereto. Typically, a suitable fragment will be 20, 30 or 40 nucleotides. Suitably stringent conditions are discussed above.

In a second aspect of the present invention there is provided a promoter comprising at least the SA responsive element from -247bp to -132bp of Figure 6. In particular, chimeric promoters may be produced which have the desired expression characteristics of the native plant promoter, and the ability to induce expression in response to SA conferred by the presence of the SA responsible element of Figure 6.

Such chimeric promoters may be produced using standard techniques or in recombinant DNA technology.

In a third aspect of the present invention, there is provided two or more inducible pathogenesis related protein promoter sequences according to the first aspect of the invention, arranged in series. The resulting promoter multimer may comprise a combination of any two or more of the preferred promoter sequences of the first

aspect of the present invention. Any number of promoter sequences may be arranged in series to produce a multimer, depending upon any size and stability constraints of the expression system, and the desired level of gene expression. Preferably, the multimer comprises at least 2, at least 5, or, most preferably at least 5 7 promoter sequences. The promoter sequences of the multimer may linked directly to one another, or via intervening linking sequences. The intervening linking sequences may be of any suitable length to allow efficient functioning of the multimer, and be derived from foreign DNA. Preferably, the multimer includes at least one promoter sequence which comprises the minimal promoter -132bp 10 sequence shown in Figure 6. Where only one of the two or more promoter sequences comprises the minimal -132bp sequence shown in Figure 6, it is preferable that this sequence is positioned closest to the gene to be expressed. In a most preferred embodiment of this aspect of the invention, there is provided a series of fragments of the promoter sequence shown in Figure 6, operably linked to the 15 minimal -132bp promoter sequence of Figure 6. In the most preferred embodiment, the fragments include the -247 to -132bp SA responsive element.

In a fourth aspect of the present invention, there is provided an amplification system, comprising a pathogenesis-related protein promoter sequence according to the first 20 aspect of the present invention. Preferably, the promoter of the present invention is operably linked to a transactivator sequence and a second promoter sequence, which is preferably the target of the transactivator sequence and is linked to DNA encoding the product of interest. Systems that can be used to amplify gene expression have been described in WO98/05789 and Moore *et al.*, *PNAS* 95 379-381 (1997). 25 Examples of amplification systems which do not involve transactivators include the mRNA viral replicase based system (Mori *et al.*, *FEBS. Letters*, 336:171-174 (1993)), where the promoter of the present invention is operably linked to a viral replicase and a second gene, where the gene transcript is amplified by the replicase.

The second gene may be anti-sense. Where the amplification system comprises a transactivator sequence, it is preferable for it to be placed downstream of the promoter sequence of the present invention, and the direction of the transcription of the promoter of this invention and the second promoter sequence to be in series or divergent. A preferred construct is shown in Figure 16, although it will be appreciated by one of skill in the art that variations of such a construct are possible which will have the effect of amplifying expression from the promoter of the present invention. In the amplification construct of this aspect of the present invention, it is envisaged that the promoter according to the first aspect of the present invention will drive expression of the transactivator. The transactivator product may then initiate multiple rounds of transcription of the desired gene via the second promoter. In this way, there will be amplification of the initial signal which activated the pathogenesis-related protein promoter of the invention. Thus, an amplification construct may allow reduced amounts of activator substances such as SA or BTH to be used, while maintaining high expression of the desired gene. In a further embodiment, it is envisaged that the multimer according to the second aspect of the invention may comprise part of an amplification system.

Transactivator sequences are known in the art, and any suitable one may be used for the purpose of the present invention. The transactivator sequences may be natural or synthetic. In a preferred embodiment, the transactivator is LhG4, which consists of the mutated *E. coli* lac I gene fused to the transcriptional activator domain of Gal4 from yeast. The second promoter sequence may be any which is activatable by the transactivator. Where the transactivator is LhG4, the preferred promoter is pOP910. This is a minimal CaMV promoter with two rounds of binding sites for the LhG4 protein. Other transactivators include the Tet transactivator, in combination with the pTop10 promoter (Wienmann *et al.*, *Plant Journal* 5 559-569 (1994)).

In a fifth aspect of the present invention, there is provided a promoter, multimer or amplification system according to the previous aspects operably linked to a DNA sequence encoding a product of interest. The DNA may encode a protein of interest, or a product able to regulate the production of a protein of interest. Proteins of interest include products sought to be harvested from a plant or plant cells in culture, products which may be expressed in the plant and alter the characteristics of the plant or plant cells in culture, products involved in the regulation of certain plant traits, and products such as marker genes. In particular, DNA encoding products which provide or enhance a beneficial feature of the transgenic plant are preferred. For example, the nucleic acid may encode proteins or antisense RNA transcripts in order to promote increased food values, higher yields, pest resistance, disease resistance, artificial male sterility (for example: barnase or PR-glucanase), female sterility, or flower and fruit ripening control. Representative nucleic acids of interest include for example, a bacterial dap A gene for increased lysine; Bt-endotoxin gene or protease inhibitor for insect resistance; lytic peptide genes for disease resistance, bacterial or plant EPSP for resistance to glyphosate herbicide (US 4, 940,835; US 5,188,642; US 4,971,908; US 5,145,783; US 5,312,910; US 5,633,435, US 5,627,061, US 5,310,667; WO 9704103), bacterial or plant HPPD (WO9638567, WO9802562) for resistance to HPPD-inhibitors herbicides (for example diketole or isoxazoles), chitinase or glucanase, 1,3-B-glucosidase for fungicidal properties. Further, a DNA sequence may be introduced to act as a genetic tool to generate mutants or to assist in the identification, genetic tagging or isolation of genetic sequences. Examples of nucleic acids useful for modifying quality include: genes for starch biosynthesis or degrading enzymes (for example starch synthases, starch branching enzymes) grain storage protein genes (for example sub-units protein of glutenin, gliadins or hordeins), or genes linked to the grain hardness.

The promoter-gene construct of the fifth aspect of the invention may further comprise a marker gene to allow monitoring of the expression of the heterologous DNA.

Preferably, a marker gene is operably linked to the promoter, multimer or

amplification system of the invention, in series with the heterologous DNA encoding a product of interest. Induction of the promoter, multimer or amplification system will result in expression of the marker gene in the transformed cell or plant, thus enabling one to assess the level of induction of the product of interest easily without the need to harvest or destroy the whole or part of the plant, or culture of plant cells. Any suitable marker gene may be used. Examples include beta-glucuronidase, luciferase or green fluorescent protein.

The promoter-gene construct may also comprise additional regulatory sequences required for the efficient expression or targeting of the gene product. For example, 3' transcription regulation signals such as polyadenylation signals may be provided, as may any other regulatory sequence such as enhancers. Preferred 3' polyadenylation signals are derived from the Cauliflower Mosaic Virus 35S gene, although one skilled in the art would appreciate that other 3' polyadenylation signals could be used. The addition of a transit peptide sequence may be desired where the product is to be secreted from the cell.

The recombinant or isolated DNA according to any one of the aspects of the present invention may be in the form of a vector. The vector may be a plasmid, cosmid or phage. The vectors may be introduced directly into plant cells, using known methods in the art or may first be cloned in bacteria such as *E. coli*, before introduction into the plant cell. Where the vectors are to be cloned in microbial host cells, it is preferable that the vector further comprises one or more marker genes to enable the selection of transformed or transfected microbial cells harbouring the vector construct comprising the heterologous DNA. Sufficient start and stop signals, and regulatory sequences to allow expression of the heterologous DNA and/or marker gene in the microbial cell may also be included.

According to a sixth aspect of the present invention there is provided a host cell transfected or transformed with DNA described above. The host cell may be a plant

cell or a microbial cell. The present invention also provides a transgenic plant cell culture, of a monocotyledon or dicotyledon, transformed with a promoter, multimer or amplification system of the present invention, preferably operably linked to a heterologous gene. Transformation methods are described below. The transgenic

5 plant cell culture may be used to generate whole plants, and thus in a further aspect of the present invention there may be provided transgenic plants, seeds and propagating material, e.g. propagated shoots comprising DNA according to the invention. Preferred plants or parts or cells thereof for transformation include rice, maize, wheat, barley, sorghum, sugarcane, tobacco, rapeseed, sunflower, soybean, cotton, clover, and

10 beans as well as sugar beet, potato, vegetables such as tomato, melon, cabbages, lettuce, carrot, beans, paprika and peppers.

The DNA of the present invention may be prepared using any convenient method involving coupling together successive nucleotides, and/or ligating oligo and/or poly-

15 nucleotides, including *in vitro* processes. Recombinant DNA technology remains the method of choice.

The DNA of the present invention may be introduced into plant cells using standard methods of the art. Preferably, DNA is transformed into plant cells using a dis-

20 armed Ti-plasmid vector and carried by *Agrobacterium*, by procedures known in the art. Alternatively, the foreign DNA may be introduced directly into plant cells using a microprojectile apparatus, or any other physical delivery system. The latter techniques are preferable where *Agrobacterium* is ineffective for stable transformation, for example in the transformation of cereal plant cells. Preferably,

25 the transformation vector will comprise a cloning site or a multicloning site for the insertion of genes or other DNA (referred to herein as passenger genes) to be transferred to plant cells. The passenger genes or other DNA may be under the control of a promoter which differs in its expression characteristics from the promoter of the invention. For example, the passenger gene may be a marker gene

under the control of a different promoter to that of the invention, such that when the construct is expressed in the plant, the marker gene may be expressed according to the characteristics of the promoter to which it linked, and not limited to the expression pattern of the promoter of the invention. Any suitable techniques which would allow for the stable incorporation of the DNA of the invention within the nuclear DNA of a plant cell would also be suitable.

In a seventh aspect of the present invention there is provided a method for the identification of agents capable of regulating the expression of heterologous genes which are operatively linked to the AoPRT-L promoter. The method may comprise the steps of applying a putative agent to a DNA sample comprising the AoPRT-L promoter operatively linked to a heterologous gene, measuring the expression level of the gene and comparing expression levels against a control system. This provides an indication of the ability of the agent to regulate gene expression via the AoPRT-L promoter. Putative agents of interest may be applied to a transformed plant, cell culture, tissue sample or *in vitro* system comprising the DNA sample. Agents of interest identified in this manner are preferably those which induce expression of a gene operatively linked to the AoPRT-L promoter.

The agent may be any natural or synthetic product, for example chemical compounds such as proteins, peptides, DNA or RNA sequences or hormones and analogues thereof. The heterologous gene to which the promoter is linked may be any gene, preferably one which expresses a quantifiable product. Preferred heterologous genes for this purpose include those which encode screenable markers, for example beta-glucuronidase or green fluorescent protein.

Agents identified by the above method may also be effective in regulating expression of the AoPRT-L gene itself or genes encoding pathogenesis related proteins

equivalent or homologous to the AoPRT-L protein of *Asparagus officinalis*. The latter may include pathogenesis related proteins of the *Lilliaceae* or *Amaryllidaceae* family, or indeed of other plant families. Such agents may be effective at regulating or inducing a full or partial SAR response, thus rendering them useful as "crop protectants" for field use.

The present invention will now be illustrated by the following Examples, which refer to the accompanying drawings, in which:

FIGURE 1 shows the Nucleotide sequence of AoPRT-L cDNA together with the predicted amino-acid sequence of AoPRT-L. The sequences and positions of binding of the primers used for IPCR are indicated above the cDNA sequence and relevant enzyme restriction sites underlined.

FIGURE 2 shows sequence homologies between AoPRT-L and other PR-5 proteins

FIGURE 3 shows the accumulation of mRNA for AoPRT-L in Asparagus. Figure 3(a) shows the induction of AoPRT-L expression following mechanical isolation of Asparagus cladode mesophyll cells. Figure 3(b) shows the induction of AoPRT-L expression in chopped etiolated Asparagus seedlings and Figure 3 (c) shows the expression of AoPRT-L in SA-treated Asparagus.

FIGURE 4 shows the expression of AoPRT-L mRNA following infection of Asparagus with *Stemphyllium versicarium*.

FIGURE 5 shows a strategy for the isolation of the AoPRT-L promoter by IPCR.

FIGURE 6 shows the Nucleotide sequence of the AoPRT-L promoter. Sequences with homology to characterised promoter elements are boxed.

FIGURE 7 shows the construction of AoPRT-L-GUS constructs.

5 FIGURE 8 shows the axial expression of AoPRT-L-GUS in transgenic tobacco compared with PR-1a-GUS.

10 FIGURE 9 shows the expression of AoPRT-L-GUS in local and systemic tissues of transgenic tobacco infected with Tobacco Mosaic Virus (TMV) or *Pseudomonas syringae* pathovar *phaseolicola*. GUS activity was measured flurometrically using the substance 4-methyl umbelliferone (4-MU) formed/minute/mg protein extract.

FIGURE 10 shows the induction of AoPRT-L-GUS and PR-1a-GUS expression in transgenic tobacco treated with exogenous SA or BTH.

15 FIGURE 11 shows the expression of (a) AoPR-1-GUS and (b) AoPRT-L-GUS in wounded transgenic tobacco, and (c) expression of AoPRT-L-GUS in transgenic tobacco leaf discs following treatment with exogenous jasmonate.

20 FIGURE 12 shows the expression of AoPRT-L-GUS in leaves of transgenic tobacco root-fed with either NaCl or PEG 8000, subjected to water stress or leaf discs treated with ABA.

25 FIGURE 13 shows the expression of AoPRT-L-GUS in transgenic tobacco leaves infiltrated with hydrogen peroxide or t-butyl hydroperoxide.

FIGURE 14 shows the expression of AoPRT-L-GUS and PR1a-GUS in T0 transgenic *B. napus* leaf discs floated for 3 days on water, 1mM SA or 250µM BTH. Data are the average of 6 independent transformants / transgene.

FIGURE 15(a) is a diagrammatic representation of the AoPRT-L promoter deletions fused to the GUS reporter gene and Figure 15(b) shows their response to exogenous SA in T0 transgenic tobacco. Figure 15(c) is a diagrammatic representation showing arrangement of putative SA responsive elements in pMultAoPRT-L promoter.

5

FIGURE 16 is a diagrammatic representation of pAoPRT-L expression amplification construct pGB24.

10

In the examples, unless otherwise stated, all procedures for making and manipulating recombinant DNA were carried out using the standard techniques and protocols described in Sambrook J., Fritsch EF & Maniatis T *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory 1989.

EXAMPLES

15

Example 1 - Isolation of an AoPRT-L cDNA and characterisation of expression of AoPRT-L in *Asparagus officinalis*.

20

Single mesophyll cells isolated from *Asparagus officinalis* are capable of dedifferentiating and initiating cell division in an appropriate culture medium (Harikrishna *et al.*, (1991) *J. Exp. Bot.* 42:791-799). Large changes in gene expression occur in these isolated cells during adaptation to cell culture conditions, thus mRNA isolated from these cells can be used to obtain genes specifically induced during this adaptation. Such genes include those upregulated by wounding and stress. The construction and screening of a cDNA library made from mechanically isolated *A. officinalis* cells is described previously (W0 93/05164; Warner *et al.*, (1992) *Plant Molecular Biology* 19:555-561). One cDNA clone representing a differentially expressed mRNA was found to represent up to 0.2% of the cDNA library. DNA sequence analysis (Figure 1) showed that the mRNA encodes a putative protein of predicted mass 21.3 kDa which is homologous to thaumatin-like genes (Figure 2) and thus is a member of the PR5 gene family. Thus the gene was named AoPRT-L.

30

Analysis of the putative AoPRT-L protein sequence showed that it is most closely related to acidic secreted PR5 (a-PR5) proteins. The AoPRT-L gene product is recognised by a polyclonal antiserum raised against PR-5 and is secreted since it is found in the culture medium of Asparagus cells.

5

Northern hybridisation analysis using RNA isolated from mechanically isolated *A. officinalis* cells indicated that AoPRT-L is upregulated in mechanically isolated cladode cells (Figure 3a). In chopped etiolated *A. officinalis* seedlings (≤ 0.5 cm in length) the AoPRT-L transcript was detectable from 3 days after wounding (Figure 3b). However AoPRT-L does not behave like the wound inducible gene AoPR1 (W0 93/05164; Warner *et al.*, (1992) Plant Molecular Biology 19:555-561) in that expression is not seen in sections of chopped etiolated seedlings greater than 0.5cm in length and expression of AoPRT-L in sections of ≤ 0.5 cm extends throughout the section and is not limited to the wound site (Figure 3b).

15

AoPRT-L mRNA accumulation was observed following treatment with SA (Figure 3c) and following infection with the fungal pathogen *Stemphyllium versicarium* (Figure 4) but was not induced by the ethylene-generating compound ethephon.

20

Example 2 - The isolation of the upstream promoter regions of the AoPRT-L gene by the Inverse Polymerase Chain Reaction (IPCR).

An AoPRT-L promoter region was isolated from *A. officinalis* by the Inverse Polymerase Chain Reaction (IPCR) (Figure 5). The technique was essentially as described in (W0 93/05164; Warner *et al.*, (1993) Plant Journal 3:191-201).

25

A. officinalis genomic DNA was digested with EcoRI, religated to circularise the restriction fragments and PCR performed with the AoPRT-L specific primers of:

P1 5'-CGCGGAATTC~~CGTGTAGGTGCATTTGTTGG~~-3' (105 -86 bp) and

Eco R1

30

Sub
B6

Sub B7
P2 5'-CGCCTGCA~~AG~~CCAATCCTGGACCCTCACCG-3' (152 – 172 bp)

~~Pst 1~~

- 5 A 0.8 kb DNA fragment was obtained that hybridised with the most 5' region of the AoPRT-L cDNA. This PCR product was cloned directly into the pCR 2.1 vector (Invitrogen) using the protocols supplied with Invitrogen's TA cloning kit. The resulting construct was named pIPCR-TA. DNA sequence analysis of the PCR product confirmed the authenticity of the fragment as containing the correct upstream
- 10 promoter sequence (Figure 6).

- Examination of the AoPRT-L promoter sequence (Figure 6) reveals regions of homology with other PR promoters have been identified including a tobacco PR-2 and carrot PR-3 and PR-4-like sequences. There also appears to be a *c-myc* consensus
- 15 sequence at -344 to -339. However, the promoter contains no consensus G-box, PR-box or ABRE which suggests that pAoPRT-L will not be induced by ABA or ethylene.

Example 3 - Construction of an AoPRT-L promoter -GUS chimeric gene.

- 20 The AoPRT-L promoter was obtained from pIPCR-TA by PCR using primers designed against both the 5'- and 3' ends of the promoter with extensions to provide appropriate restriction sites for further cloning:

25 5'- GGGTACCAAGCTT~~CTT~~TATTGCGACCTGACTCTC 3'

KpnI HindIII

5'- CGCGGATCC~~GT~~TCGACCTGCAGGATTGGTTGTGTGTTGTTTT 3'

BamHI Sall PstI

Sub B8

This PCR product was then digested with KpnI and PstI and ligated into the pJIT60 vector (identical to pJIT30 (Guerineau *et al.*, (1990) Plant Mol. Biol. 15, 127-136) but with a double rather than a single 35S CaMV promoter) digested with same enzymes. The result was named p22-JIT60. The sequence encoding the β -glucuronidase reporter enzyme containing an intron (GUS (INT) in pBluescript SK- (Firek *et al.*, (1993) Plant Mol. Biol. 22: 129-142; Jefferson *et al.*, (1987) EMBO J. 6:3901-3907; Vancanneyt *et al.*, (1990) Mol. Gen. Genet 220: 245-250) was cloned into p22-JIT60, behind the AoPRT-L promoter sequence, by digesting with BamHI and EcoRI to give the construct p22-GUS(INT) JIT60. Finally the entire AoPRT-L promoter-GUS fragment was released from p22-GUS(INT) JIT60 using KpnI and XhoI and ligated into the binary vector pBIN19 (Bevan (1984) Nuc. Acids Res. 22: 8711-8721) which had been digested with KpnI and Sall to give p22-GUS(INT) Bin19 (Figure 7).

The GUS gene is a convenient reporter gene whose expression can be monitored easily. It should be understood that in this and in the following examples GUS can be replaced by any gene of interest, to allow chemical-induction of the gene of interest.

Example 4- Developmental expression of the AoPRT-L promoter in transgenic tobacco.

The AoPRT-L promoter construct (p22-GUS(INT) Bin19) was transformed into *Nicotiana tabacum* (cv Samsun) using standard *Agrobacterium tumefaciens*-mediated transformation techniques (Draper *et al.*, (1988): Plant Genetic Transformation and Gene Expression - A Laboratory Manual. Blackwell Scientific Publications, Oxford, UK). The expression of the GUS gene driven by the AoPRT-L promoter was analysed using assays described previously (Draper *et al.*, (1988) *supra*).

Histochemical analysis demonstrated that, in untreated plants, AoPRT-L promoter-driven GUS is expressed only in sepals and at petiole-stem junctions (leaf axils) (Figure 8). Additionally, histochemical staining of roots close to the crown (base of

the stem) is occasionally observed. Thus the promoter exhibits minimal developmental activity in comparison to other published promoters.

Example 5 - Non-Systemic induction of AoPRT-L promoter-driven GUS

expression in pathogen-challenged transgenic tobacco.

AoPRT-L-GUS-transformed Samsun tobacco plants were infected with Tobacco Mosaic Virus (TMV) by abrading the surface of a single leaf with a mixture of virus and carborundum as previously described (Bi *et al.*, (1995) Plant J. 8:235-245). TMV induces an N-gene-dependent hypersensitive response characterised by the appearance of lesions (areas of hypersensitive cell death) on the infected leaf. Inoculation of Samsun tobacco with TMV also induces SAR and the systemic accumulation of endogenous PR-1a (Bi *et al.*, (1995), *supra*). At various time points after infection, leaf discs were cored from lesions (these cores also contained non-HR tissue directly abutting the lesion, from uninfected inter-lesion tissue on the same leaf and from a non-infected systemic leaf on the same plant). GUS activity in these discs was measured fluorimetrically. GUS activity was found to be elevated in the inoculated leaf in tissue undergoing HR (or tissue directly adjoining HR lesions) but not in inter-lesion or systemic tissue (Figure 9).

Samsun tobacco plants harbouring the AoPRT-L promoter fusion with GUS were infected with *Pseudomonas syringae* pathovar *phaseolicola* (2×10^8 per ml) by inoculation of the intercellular leaf spaces of a single leaf as previously described (Bi *et al.*, (1995), *supra*). *Pseudomonas syringae* pathovar *phaseolicola* induces a non-host hypersensitive response at the site of infiltration. This treatment also induces systemic accumulation of endogenous PR-1 proteins (Bi *et al.*, (1995), *supra*). At various time points after infection, leaf discs were cored from infected leaves and from non-infected systemic leaves on the same plant. Samples taken from infected leaves included samples from the infiltration site (destined to undergo, or undergoing, hypersensitive cell death) and from non-infiltrated tissue close to the inoculation site on the same leaf. GUS activity in these discs was measured. In this case GUS activity

was only detected in samples taken from tissue adjacent to the HR lesion (Figure 9). No activity was detected within the tissue undergoing HR or in systemic tissue. This suggests that it is the tissue surrounding TMV lesions rather than the lesion itself which accounts for the activity observed in TMV-infected tobacco. The higher activity observed following TMV infection is possibly due to the approximately 10-fold higher levels of SA observed following TMV challenge when compared to *Pseudomonas syringae* pathovar *phaseolicola*.

These data indicate that, unlike PR-1a, the AoPRT-L promoter is only activated in tissue close to hypersensitive response lesions and is not induced systemically.

Example 6 - Induction of AoPRT-L promoter-driven GUS expression in SA- and BTH-treated transgenic tobacco.

AoPRT-L-GUS-transformed Samsun tobacco plants were root-fed with increasing concentrations of SA for 3 days after which time leaf cores were taken and GUS activity was measured flurometrically. Substantial induction of the AoPRT-L promoter-GUS fusion was observed at 1mM SA, with little induction at 0.1mM SA (Figure 10a). Thus, the AoPRT-L promoter-GUS fusion is an order of magnitude less sensitive to SA than PR-1a -GUS which is clearly induced at 10-100 μ M SA (Bi *et al.*, (1995), *supra*; Mur *et al.*, (1996) Plant J. 9:559-571). Treatment with 1mM SA showed a clear time-dependent induction of the AoPRT-L promoter-GUS fusion compared to the inactive SA analogue 4-hydroxybenzoic acid (4hBA), observable 1 day after treatment commenced.(Figure 10b). SA induction of AoPRT-L-GUS-transformed plants gives about 1/3 of the GUS activity seen in pPR-1a-GUS-transformed tobacco. (Figure 10c).

AoPRT-L-GUS-transformed Samsun tobacco plants were sprayed with 1mM SA or 20 μ M BTH (applied in a 0.01% sapogenat solution used as a wetting agent). Plants were sprayed once; allowed to dry and then sprayed again on the same day (day 0). After 3 or 6 days leaf cores were taken and GUS activity was measured using a

standard fluorimetric assay. BTH proved to be as effective an inducer of GUS expression driven by the AoPRT-L promoter as was SA (Figure 10d). Leaf cores from tobacco harbouring the AoPRT-L promoter fusion with GUS were floated for 3 or 6 days on water containing increasing concentrations of BTH after which time GUS activity in the leaf discs was measured fluorometrically. BTH-induced GUS activity is observed with the use of 1.25 μ M BTH (Figure 10e).

These data indicate:-

- 1) the AoPRT-L promoter-GUS fusion is inducible by SA in transgenic tobacco.
- 2) That the AoPRT-L promoter-GUS fusion is an order of magnitude less sensitive to SA concentration, and shows approximately 1/3 of the induction compared to a PR-1a promoter-GUS construct.
- 3) BTH is an effective inducer of the AoPRT-L promoter-GUS fusion when applied as a foliar spray or *in vitro*.

Example 7 - Wounding or the wound signal jasmonic acid, ABA and osmotic stress fail to induce AoPRT-L promoter-driven GUS expression in transgenic tobacco.

Tobacco plants harbouring either the AoPRT-L promoter fusion with GUS or an AoPR-1-GUS construct were wounded by one of 3 methods. The leaf lamina was either crushed with forceps, sliced with scissors or subjected to a combination of crushing and puncturing by striking the leaf with a meat tenderising mallet (hammer). After 1, 2, 3, 6 or 8 days leaf cores were taken from the damaged tissue and GUS activity measured. AoPR-1 is a wound-induced gene expressed at wound sites in chopped etiolated *A. officinalis* seedlings. The AoPR-1 promoter-GUS construct is also wound-inducible when introduced into tobacco (Warner *et al.*, (1994) *Plant J.* 6:31-43; Mur *et al.*, (1996) *Plant J.* 9:559-571). AoPR-1 promoter-driven GUS

activity is elevated by all 3 wounding treatments with enhanced GUS activity observable at 1-2 days following treatment (Figure 11a). In contrast, no increase in AoPRT-L promoter-driven GUS expression was observed with any wounding treatment with the exception of a modest increase at day 8 following crushing with forceps which is likely not to be due to wounding since at this stage the tissue is extremely desiccated (Figure 11b).

Wounding of tobacco leaves (using the hammer method) or infection of tobacco with *Pseudomonas syringae* pathovar *phaseolicola* induces local accumulation of the wound-related phytohormone jasmonic acid (JA) (Kenton *et al.*, submitted; Mur *et al.*, (1997) Trends in Microbiol 5:297-300). Leaf cores from tobacco harbouring the AoPRT-L promoter fusion with GUS were floated for 3 days on water containing increasing concentrations of JA after which time GUS activity in the leaf discs was measured using a standard fluorimetric assay. Compared with flotation on 1mM SA, JA failed to induce any increase in AoPRT-L promoter-driven GUS activity at any concentration tested (Figure 11c).

These data indicate that, compared to expression of a wound-inducible promoter-GUS fusion (AoPR-1 promoter-GUS), the AoPRT-L promoter-GUS construct is essentially insensitive to mechanical damage. In addition, the AoPRT-L promoter-GUS is not induced by the known wound signal JA and by ethylene.

Example 8 - Salt and water stresses, and the water stress-related hormone abscisic acid (ABA) fail to induce substantial expression of AoPRT-L-promoter-GUS activity in transgenic tobacco.

Since AoPRT-L belongs to class 5 family of PR proteins which also contains drought-induced genes such as those encoding osmotins, the response to a number of water-related stresses was examined. Neither a high concentration of salt or 20% PEG 8000, both of which induce expression of osmotin or osmotin-like genes in several species,

induced expression of the AoPRT-L-promoter-GUS construct in transgenic tobacco (Figures 12a and 12b). A slight increase in expression was observed when water was withheld for 20 days from tobacco harbouring the AoPRT-L-promoter-GUS construct, by which time the plants were severely wilted (Figure 12c). Similarly, a high concentration of ABA induced a slight increase in AoPRT-L-promoter-GUS in leaf discs of transgenic tobacco (Figure 12d). Two points should be emphasised. 1) The level of drought or ABA induction is typically an order of magnitude lower than that observed using SA. 2) Both dehydration and ABA treatment induce substantial protein loss in tobacco, thus the apparent increase in GUS activity may be an artefact of differential sensitivity of GUS (compared with some other major leaf proteins) to the mechanism of protein loss.

These data suggest that the AoPRT-L promoter is substantially insensitive to water-related stresses and that any low-level expression which might occur is likely only in severely damaged plants.

Example 9 - The AoPRT-L promoter-GUS construct is insensitive to pro-oxidants.

The observation that AoPRT-L promoter-driven GUS activity is elevated in tissue close to the site of pathogen challenge suggests a role for SA as a causal agent of induction. However, the production of reactive oxygen species (ROS) during plant-pathogen interactions has received considerable interest in recent years. Briefly, recognition of a pathogen results in the production of a burst of H_2O_2 . Currently the most popular model for this 'oxidative burst' is that H_2O_2 derives from the dismutation of superoxide produced by a cell-surface NADPH oxidase. Several defence-related genes (including AoPR1) are directly sensitive to H_2O_2 (Bi *et al.*, (1995) *supra*). In addition, high levels of H_2O_2 are capable of inducing SA synthesis (Neuenschwander *et al.*, (1995) *Plant Journal* 8:227-233; Summermatter *et al.*, (1995) *Plant Physiology*

108:1379-1385). Finally, ROS accumulation is also a feature of chilling, ozone and UV stress and elevated ROS levels are also found in senescent tissue.

In order to test whether oxidative stress is likely to induce high levels of AoPRT-L promoter-driven gene expression, H₂O₂ was infiltrated into leaf panels of transgenic tobacco harbouring the AoPRT-L-promoter-GUS construct (Figure 13a). The concentrations of H₂O₂ used have been shown to induce AoPR1-GUS expression in tobacco (Bi *et al.*, (1995) - see Example 5). H₂O₂ failed to induce AoPRT-L-promoter-GUS expression over the concentration range tested. However, H₂O₂ has a limited half-life (around 10 mins - Levine *et al.*, (1994) *Cell* 79: 583-593) in the apoplast, thus the experiment was repeated using a stable peroxide, *t-butyl*-hydroperoxide (Figure 13b). Again, no GUS expression was detected despite the severe visible tissue damage which occurred at higher concentrations of *t-butyl*-hydroperoxide (in contrast to H₂O₂ which produced no visible symptoms).

These data suggest that, unlike PR-1a (Bi *et al.*, (1995) - see Example 5; Neuenschwander *et al.*, (1995) *Plant Journal* 8: 227-233), AoPRT-L promoter-driven gene expression is unlikely to be encountered even under conditions of high ROS stress or in the presence of ROS-mediated tissue damage.

Example 10 - The AoPRT-L promoter is induced by SA and BTH in *Brassica napus*.

The AoPRT-L-GUS fusion in pJIT60-P22 GUS-int was transferred as a SstI- XhoI fragment into SstI, SalI - cut pTZ19 (Pharmacia) forming pGB4. The AoPRT-L-GUS gene was then cloned as a HindIII fragment into the HindIII site of the binary vector SCV-nos nptII (W0 96/30529) forming pGB4-SCV, in which the direction of transcription of AoPRT-L is the same as that of the nptII gene. pGB4-SCV was transferred to the agrobacterial strain pGV2260 and transformed *B. napus* plants produced by agrobacterial transformation essentially as described in Moloney *et al.*,

(1989) *Plant Cell Reports* 8: 238-242. Transformed plants exhibited similar levels of GUS activity when treated with 1mM SA or 250 μ M BTH (Figure 14). AoPRT-L-GUS and PR1a plants also exhibited similar levels of GUS activity on SA or BTH induction (Figure 14)

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Example 11 - The AoPRT-L promoter is induced by SA and BTH in *Zea mays*.

Maize plants containing AoPRT-L -GUS were produced by biolistic transformation of callus material with pJIT60-P22 GUS int. The transformed plants exhibited GUS activity when treated with SA or BTH.

10

Example 12 - Identification and multimerisation of an SA/BTH responsive element in the AoPRT-L promoter.

A series of 3 AoPRT-L 5' promoter deletion - GUS fusion constructs were constructed using the following primers designed to regions of the AoPRT-L promoter (Figure 15a):-

15

5' GCGAAGCTTCCATGTCATGAGAGAAGCAC 3' (-361bp)

HindIII

20

5' GCGAAGCTTTTGGAAACTGAATACCTACA 3' (-247bp)

HindIII

5' GCGAAGCTTACAAAGGCTTAGACTTTCCA 3' (-132bp)

HindIII

25

Each of the above primers, in conjunction with the primer below, was used in a PCR reaction with p22-JIT60 as template:-

5' GGGATCCGTCGACCTGCAGATTGGTTGTGTGTTGTTTTG 3'

BamHI

Sall

PstI

30

The PCR products were cloned as HindIII, BamHI fragments into HindIII, BamHI-cut p22- GUS (INT) JIT60. The resulting pAoPRT-L -GUS-CaMV polyA chimeric

genes were cloned as KpnI, XhoI fragments into KpnI, Sall – cut pBin19 and transformed into Samsun tobacco. The GUS activity of transformants was measured after induction of leaf discs with 1mM SA. A significant reduction of activity was observed after deletion of up to -132 bp (Figure 15b). Thus the SA responsive element
 5 lies between -247 bp and the putative CAT (-50 -47) and TATA box at -64 to -57 bp.

In order to construct an AoPRT-L promoter that has higher expression the region -247 bp to -133 bp was amplified from p22-JIT60 and placed twice in front of a -247 bp AoPRT-L promoter. This AoPRT-Lx3 promoter was constructed as follows:-

10 The primers below were used to PCR the 0bp to -247bp AoPRT-L promoter from p22-JIT60.

5'- TCTAGGTACCCTTTGCGTGGTTCGACTTGGAACTGAATACCTAC-3'

KpnI

Sall

5' GGGATCCGTCGACCTGCAGATTGGTTGTGTGTTGTTTTG 3'

BamHI Sall PstI

This was cloned as a KpnI, PstI fragment into pUC19. The -133bp to -247bp
 20 pAoPRT-L region was amplified with the primers:-

5' TCTAGGTACCCTTTGCGTGGTTCGACTTGGAACTGAATACCTAC 3'

KpnI

Sall

25 5' GAAAGTCTAAGCCTCGAGGGAATAAGGTACGAGTTCGTGGAC 3'

XhoI

This fragment was cloned as a KpnI, XhoI fragment between the KpnI and Sall sites of the pUC19-derived plasmid forming pAoPRT-Lx2. The -133bp to -247bp
 30 pAoPRT-L fragment was then cloned as a KpnI, XhoI fragment between the KpnI and

Sall sites of pAoPRT-Lx2 forming pAoPRT-Lx3 (figure 15c). Next the AoPRT-Lx3 promoter was cloned as an KpnI, PstI fragment into KpnI, PstI-cut p22-JIT60 forming pAoPRT-Lx3-JIT60. The pAoPRT-Lx3 was transferred from pAoPRT-L-x-JIT60 as a KpnI, BamHI fragment into KpnI, BamHI-cut p22- GUS (INT) JIT 60. Finally the
 5 resulting pAoPRT-Lx3 –GUS-CaMV poly A chimeric gene was cloned as KpnI, XhoI fragments into KpnI, Sall – cut pBin19 and transformed into samsun tobacco. The resulting plants exhibit a significantly greater level of GUS activity on SA or BTH induction than the equivalent AoPRT-L-GUS plants.

10 **Example 13 - Amplification of expression of the AoPRT-L promoter by the use of a transactivating system.**

A way of increasing the activity of the AoPRT-L promoter whilst retaining tight regulation of the promoter is to use a system that will amplify expression of the AoPRT-L promoter. An amplification system was built using a transactivator
 15 (described in WO98/05789 and Moore *et al.*, (1997) *Proc. Natl. Acad. Sci. USA* **95**, 379-381). The AoPRT-L promoter is linked to a synthetic transactivator (LhG4) and the target promoter of the transactivator (pOP) to the gene to be expressed. LhG4 consists of a mutated *E. coli* lacI gene fused to the transcriptional activator domain of the GAL4 from yeast. pOP910 is a minimal 35S CaMV promoter with two binding
 20 sites for the LhG4 protein. Since the transactivator can initiate multiple rounds of transcription from pOP, there is amplification of the initial signal that activated the AoPRT-L promoter.

The amplification system was constructed by the following steps. The pOP910
 25 promoter was excised from pX-910TAG (K. Palme, Max Planck Institut für Zuchtungsforchung, Koln, Germany) as a SstI-NcoI fragment and cloned into SstI,NcoI - cut pDH68 (pDH68 consists of a Pea Plastocyanin promoter (Pwee and Gray (1993) *Plant J.* **3**, 437-449) linked to an intron GUS gene (D. Twell, Leicester University) cloned between the SstI and EcoRI sites of the vector pJIT30 (Guerineau
 30 *et al.*, (1990) *Plant Mol. Biol.* **15**, 127-136)). The resulting pOP-GUS int -CaMV

polyA chimeric gene was transferred as a SstI (rendered blunt), XhoI fragment into the EcoRV, XhoI -cut cloning vector pIC19H (Marsh *et al.*, (1984) *Gene* 32, 481-485).

From this plasmid the chimeric gene was recovered as a Sall, XhoI fragment and cloned into Sall-cut pNos nptII-SCV forming pWP320-SCVA. To construct a fusion

5 of pAoPRT-L to LhG4 the AoPRT-L promoter was first transferred as an HindIII, PstI fragment from pGB4 into HindIII, PstI - cut pBluescript KS+ forming pGB21.

pGALA (I, Moore, University of Oxford, UK) contains the LhG4 gene linked to a CaMV poly A terminator. This gene was excised as a KpnI, PstI fragment and cloned between the KpnI and PstI sites of the vector pT7Blue2 (Novagen) forming pGB22

10 The Kpn (rendered blunt), NotI fragment of pGB22 was then cloned into the SmaI, NotI sites of pGB21 forming pGB23. The resulting pAoPRT-L-LhG4 fusion was transferred as an Sal fragment into Sall-cut pWP320-SCVA forming pGB24 (Figure 16).

15 Tobacco and *B. napus* plants containing the pGB24 T-DNA region exhibit a significantly greater level of GUS activity on SA or BTH induction than equivalent AoPRT-L-GUS plants.

20